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13. ABSTRACT (Maximum 200 Words)

Purpose: Identification and characterization of proteins involved in intrinsic negative feedback loops autoregulating the transforming c-ErbB2/EGF Receptor activity in *vivo*.
Scope: Receptor Tyrosine Kinase (RTK) activation trigger two distinct signal transduction cascades: Mitogenic Ras dependent MAP Kinase activation and PI3 Kinase dependent Akt/PKB survival signaling. In *Drosophila*, EGF Receptor activity is subject to Ras dependent negative feedback regulation mediated by the Kekkon (Kek) family of proteins. Searching the sequence of the human genome, putative orthologs of Kekkon are identified and tested for activity in ErbB2 feedback regulation. From the PI3K - Akt/PKB signaling branch, no such genes are known so far. To this end, a cell based genome wide screen employing double stranded RNA interference (dsRNAi) as well as an analysis of Akt mediated transcriptional response using DNA microarrays covering the whole genome have been initiated.
Progress: 12 putative human Kek orthologs have been identified and a real-time RT-PCR assay analyzing their transcriptional response to ErbB2 activation has been established. Phospho-specific Antibodies against *Drosophila* Akt and S6 Kinase have been characterized and have been used for a initial test of a cell based dsRNAi screen. DNA Microarrays covering the *Drosophila* genome and the required application protocols have been generated.

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Introduction

Background

Recent advances in understanding the molecular alterations underlying breast cancer development and progression highlight the importance of the ErbB receptor tyrosine kinases family; members of the Epidermal Growth Factor Receptor (EGFR) superfamily (1,2). The activity of the c-ErbB2 receptor tyrosine kinase (RTK) has been linked to the oncogenic process in *vivo* (3). In particular, 20 to 30% of the invasive breast cancers are marked by *c-erbB2* (HER-2/neu) gene amplification and/or overexpression (4,5).

The activation of Receptor Tyrosine Kinases (RTK) like c-ErbB2 trigger two distinct signal transduction pathways: Mitogenic Ras dependent MAP Kinase activation and PI3 Kinase dependent Akt/PKB survival signaling (6,7,8,9). Recent evidence suggests that signaling mediated by both of these branches can protect breast cancer cells from apoptosis as well as stimulate cell proliferation (10).

Studies performed in *Drosophila* have revealed a tight temporal and spatial control of EGFR pathway activity by negative feedback circuits (11,12). Based on the significant molecular conservation of the signal transduction machinery between man and the fly, it was hypothesized that negative feedback circuits regulating c-ErbB2 activity are present in humans. Additionally, this intrinsic feedback control might be inactivated by mutation in breast cancer patients, thereby contributing to the poor clinical outcome of c-ErbB2 positive breast cancers.

Goal

The purpose of this work is the identification and characterization of proteins involved in intrinsic negative feedback loops autoregulating c-ErbB2/EGF Receptor activity in *vivo*. In *Drosophila*, EGF Receptor activity is subject to Ras dependent negative feedback regulation mediated by the Kekkon (Kek) family of proteins (11,12,13). Failures of this negative feedback circuit result in ectopic activation of the *Drosophila* EGFR, finally leading to the death of the organism (13). Searching the sequence of the human genome, putative orthologs of Kekkon are identified and tested for activity in ErbB2 feedback regulation.

Currently, no genes involved in feedback regulation of the PI3 Kinase - Akt/PKB signaling branch have been described yet, although the presence of feedback regulation has been evident in *Drosophila* (14, 15). In order to identify regulators of Akt activity, a cell based genome wide screen employing double stranded RNA interference (dsRNAi) has been initiated. Furthermore, to search for transcriptional targets which might be involved in the regulation of Akt/ PKB activity, transcriptional profiling experiments employing using DNA microarrays covering the whole genome are currently in progress.

Significance

Diagnosis of c-ErbB positive breast cancer correlates with a poor overall survival rate of patients. This identifies the family of ErbB RTKs as an important therapeutic target. Proteins that down-regulate the transforming activity of ErbB Receptor Tyrosine Kinases should either represent interesting drug targets or potentially act as efficient therapeutics on their own.

Body

A search for human orthologs of *Drosophila* Kekkon proteins

The five known *Drosophila* Kekkon proteins constitute a small family of Type I transmembrane proteins. The commonality of their extracellular parts is based rather on the stereotypical arrangement of domains than strict homology on primary amino acid sequence level. A N-terminal Leucine rich domain (LRR N-term) is followed by five to seven "classical" LRR motives, trailed by an IgG domain of the C2 class. PSI BLAST searches of the human genome using the extracellular parts of the *Drosophila* Kekkons as a query have been performed, and the outcome has been analyzed for the presence of the stereotypical Kekkon domain arrangement (16, 17).

Two broad categories of human orthologs are found, one group being distinct by the presence of a Fibronectin type III Domain.

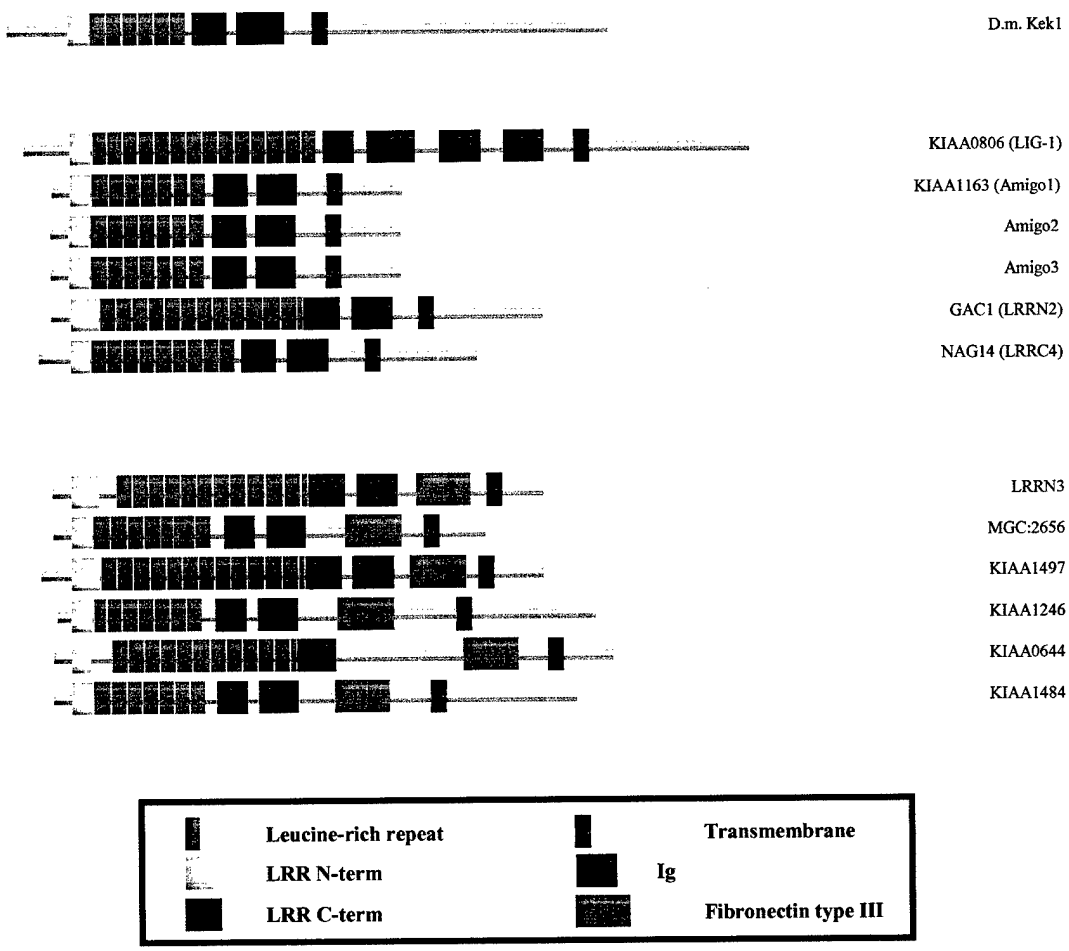

Figure 1: Domain structure of the putative human Kekkon orthologs.

Twelve candidate protein sequences for human orthologs of *Drosophila* Kekkon have been isolated. Their N termini contain the Kekkon-typical domain structure of LRR N-term, seven to fourteen LRR motives and an IgG C2 domain. The lower group shown here harbors an additional Fibronectin type III domain.

For most of the protein sequences, only Est sequence data has been published. However, there are publication reporting the cloning of Lig1 (18, 19), Amigo1, 2 and 3 (20) and GAC1 (21). Furthermore, Amigo1 has been suggested to possess properties as a neuronal cell adhesion molecule and might be required for the fasciculation of neurites (20) while GAO1 has been reported to be overexpressed in malignant gliomas (21).

Establishment of a quantitative real time RT-PCR assay

Choosing the non-conserved intracellular domains of the putative human kekkon cDNAs as a target, primers have been designed in order to measure mRNA quantities in tissue culture experiments as a well as in breast cancer tissue samples (22). Gene specific reverse transcription is combined with real-time detection and quantification of the PCR product using SYBR Green chemistry and a Lightcycler (Roche) (23). The analysis of ErbB2 overexpression mediated elevation of putative hkeks mRNA is ongoing.

Identification and characterization of a *Drosophila* phospho-specific Akt Antibody

In collaboration with Cell Signaling, Beverly, MA, an antibody against Serine 505 of *Drosophila* Akt (homologous to Ser 473 of human Akt1) has been generated (P-dAkt^{Ser505}). Ser 505 phosphorylation has been reported to be required for Akt activation (24). The specificity of the antibody was shown by Western Blot on extracts of Insulin stimulated SL2 cells in comparison to non-stimulated cells and extracts of SL2 cells subjected to dsRNAi mediated depletion of Akt as controls. In addition, the P-dAkt^{Ser505} antibody recognizes endogenous levels of phosphorylated Akt in cultured *Drosophila* SL2 and KC_{167} cells activated by Insulin stimulation in an immunohistochemical stain. Unstimulated and Insulin stimulated cells treated with the PI3 Kinase inhibitors LY294002 and Wortmannin were used as negative controls (Figure 2).

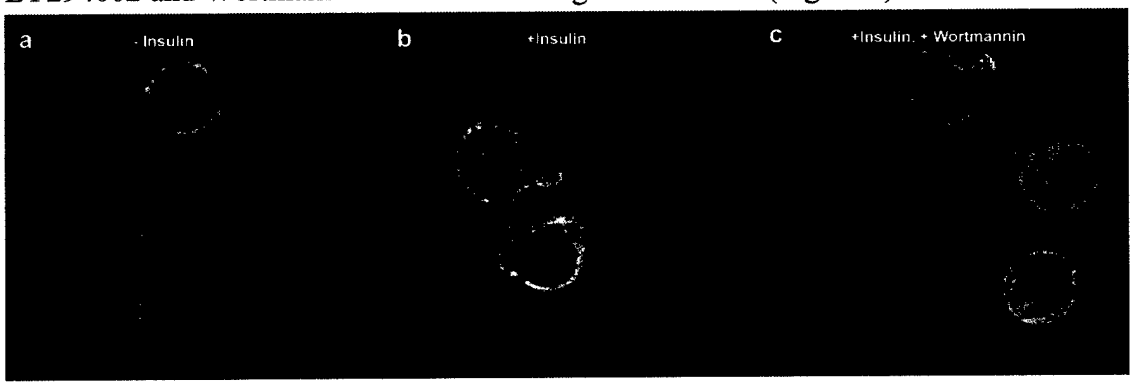

Figure 2: Insulin induced PI3 Kinase dependent dAkt phosphorylation in *Drosophila* SL2 cells

Cultured *Drosophila* SL2 cells were either serum starved for 12 hours (a) or treated for 30 minutes with Insulin in the absence (b) or presence (c) of 400nM Wortmannin as indicated. Subsequently, cells have been fixed and stained with anti-alpha Tubulin (shown in green) and anti P-dAkt^{Ser505} (shown in red). Phosphorylation of dAkt is strongly detected after Insulin stimulation and absent when PI3 Kinase, an essential upstream component of Akt activation, is inhibited by Wortmannin. Please note that anti P-dAkt^{Ser505} stains the cell perimeter, presumably the cytoplasmic membrane, when SL2 cells are stimulated with Insulin, but not when treated with Wortmannin and Insulin. This is in agreement with the translocation from the cytoplasmic compartment to the inner cell membrane as a required step for Akt phosphorylation and activation (24).

Initiation of a genome wide cell based dsRNAi screen for regulators of Akt

A unique collection of 21.500 dsRNAs, covering all genes annotated in the *Drosophila* genome, has been established in the laboratory of Prof. Norbert Perrimon. Treatment of cultured *Drosophila* cells with gene-specific dsRNA has been shown to exclusively deplete the mRNA of the targeted gene, concomitant with a sharp decrease of the related protein level (25). The combination of treating *Drosophila* SL2 cells with dsRNA against every single gene with immunohistochemical staining against phosphorylated/activated Akt allows the systematic identification of regulators of Akt.

To this end, a high throughput protocol using 96- or 384-well microtiter plates for culturing, dsRNA treating, P-dAkt^{Ser505} antibody staining and microscopically analyzing *Drosophila* cells has been established.

As a pilot screen, 94 genes encoding for Kinases, Phosphatases, small GTPases as well as other signaling molecules have been selected and tested. *Drosophila* KC_{167} cells have been cultured for in a 96 well microtiter plate containing a single dsRNA per well. After three days cells were subjected to 12 hours of Serum starvation, followed by stimulation with 5µg/ml Insulin for 30 minutes. A well containing no dsRNA as well as dsRNA against GFP have been used as positive controls, a well containing dsRNA against Akt served as negative control. Subsequently, cells were fixed and stained with DAPI and P-dAkt^{Ser505} and anti Tubulin antibodies. Each well was analyzed by an Autoscope, a microscope with a motorized stage and automatic image acquisition software.

Among the 96 dsRNAs used, the phosphorylation status of Akt can be grouped into three classes: dsRNAs which do not have an effect and a wild type phosphorylation staining of Akt is detected (Figure 3A), dsRNAs which result in a loss of detectable Akt phosphorylation (Figure 3B), dsRNAs which cause a stronger phospho-Akt response than wild type (Figure 3C).

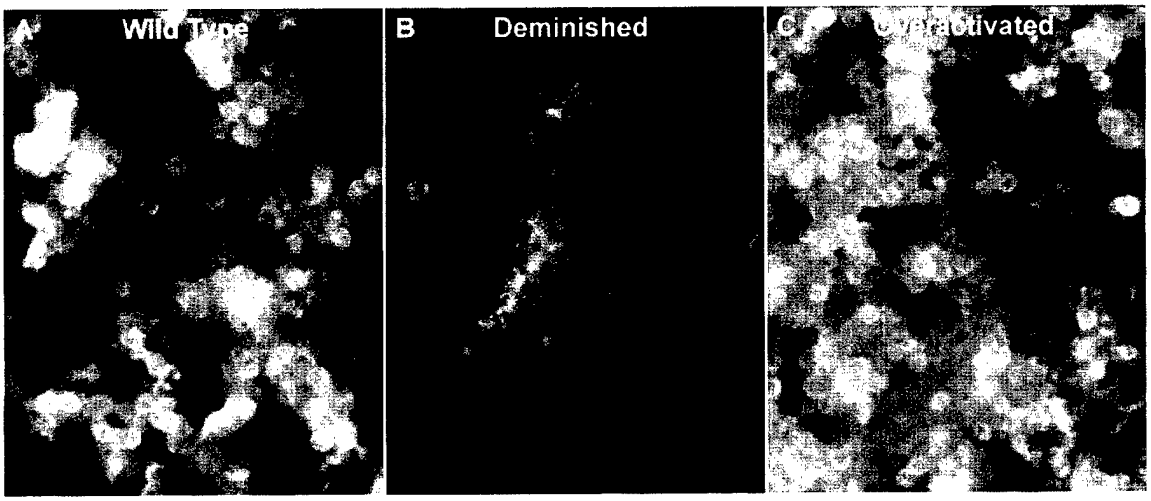

Figure 3: Three classes of Akt phosphorylation

Drosophila SL2 cells were cultured in a 96-well microtiter plate containing gene-specific dsRNAs for three days. After Serum starvation followed by Insulin stimulation, cells were fixed and stained with the nuclear dye DAPI (blue), anti alpha Tubulin (red) and anti P AktSer505 (green). Three classes of Akt phosphorylation have been found: (A) Wild type activation of Akt in response to Insulin treatment. (B) Diminished or absent Akt phosphorylation. (C) Phosphorylation of Akt above the wild type level.

The first class, wild type Akt phosphorylation, contains 83 out of 96 wells. In this group are the non-dsRNA treated cells as well as the GFP positive control. In the second class, reduced or diminished Akt phosphorylation, contains the negative control dsRNA against Akt as well as dsRNAs against JNK phosphatase Puckered and the DNA checkpoint kinase Grapes. The third group, stronger phospho-Akt response than wild type, contains the JNK Kinase Hemipterous, the non-Receptor Tyrosine Kinase Src29 and the dual-specificity phosphatase MKP-1.

Based on the findings of a JNK phosphatase and a JNK Kinase in the complementary groups of reduced and elevated Akt phosphorylation, respectively, it is tempting to speculate about a regulatory role of JNK signaling in Akt activation. Work is currently on its way to consolidate and dissect this connection.

This experiment has been the proof of principle for a genome wide dsRNAi screen for regulators of Akt activation. As the pilot screen revealed activators as well as negative regulators, the genome wide dsRNAi screen has strong potential to reveal a complete picture of Akt regulation. Active Akt signaling has been linked to the poor clinical outcome of c-ErbB2 positive breast cancer (6,7,8,9). Understanding the in- and outputs of Akt activation will lead to additional valuable therapeutic targets to fight breast cancer.

A search for transcriptional targets of Akt signaling

To search for transcriptional targets which might be involved in the regulation of Akt/ PKB activity, transcriptional profiling experiments employing using DNA microarrays covering the whole genome are currently in progress.

In collaboration with the Bio-Polymer Facility of the Department of Genetics, Harvard Medical School, the laboratory of Prof. Michelson, Brigham and Womens Hospital and the Harvard Partners Center for Genomics and Proteomics, a primer set covering all genes of the *Drosophila* genome release 1.0 (13.500 genes, (26)) has been purchased. PCR products have been amplified and spotted on GAPS coated glass slides. Protocols have been set up for fluorescent labeling of RNA pools, cDNA hybridization on glass DNA microarrays and post-hybridization data treatment. A mRNA standard containing a mixture of embryonic, larval, adult and cell line mRNA in an amount allowing approximately 70 comparative hybridizations has been produced. Values of detection limits (300.000 molecules per RNA pool = 5-10 transcripts per cell) and fold cut-off (two fold cutoff with a 99.5% confidence, 1.6 fold cutoff with 98% confidence) have been established

This *Drosophila* microarray allows the genome wide search for transcriptional targets of Akt signaling. In order to identify these genes, RNA was isolated from several time points (30 min, 1, 2 and 4 hours) of Insulin treated cultured *Drosophila* SL2 and compared to untreated, Serum starved control RNA pools.

Surprisingly, no significant changes of expression could be detected in this experimental setting. This might be due to the low-level expression of at least four out of seven Drosophila Insulin like peptides (dilps) present in Serum starved SL2 cells, which was detected in the course of these experiments.

A search for a cell line which is transcriptionally responsive to Akt signaling as well as experiments adding PI3 kinase inhibitors to the serum starvation are underway.

Key Research Accomplishments

- Human genome sequence searched for proteins orthologous to *Drosophila* Kekkon. 12 putative genes identified
- Gene specific primers to each of these genes have been synthesized, real time RT-PCR assay has been established
- A phospho-specific antibody against phosphorylated Ser505 of *Drosophila* Akt has been characterized and tested on western blotting and immunohistochemistry employing dsRNAi and chemical inhibitors.
- High throughput protocol established for screening dsRNAi treated tissue culture cells fluorescently labeled for anti-phospho-Akt in an 96 well plate tissue culture format using an autoscope
- First pilot dsRNAi screen for genes required for Akt Ser505 phosphorylation, screening a selection of dsRNAs against Kinases, Phosphatases and other signal transduction molecules
- Co-production of a *Drosophila* genome wide, PCR based DNA mircoarray
- Protocols have been set up for fluorescent labeling of RNA pools, cDNA hybridization on glass DNA microarrays and post-hybridization data treatment. Values of detection limits and fold cut-off have been established
- Experiments profiling the genome wide transcriptional response to Akt/PKB activation in progress

Reportable outcomes

None yet.

Preliminary conclusions and outlook

As the RT-PCR screening of ErbB2 stimulated tissue culture cells as well as RT-PCR screening of breast cancer tissue samples will be on its way soon, I expect to single out the significant putative human Kekkon orthologs out of the group of the twelve candidates described here.

The pilot dsRNAi screen has been the proof of principle for a genome wide dsRNAi screen for regulators of Akt activation. As the pilot screen revealed activators as well as negative regulators, the genome wide dsRNAi screen has strong potential to reveal a complete picture of Akt regulation. Active Akt signaling has been linked to the aggressiveness and poor clinical outcome of c-ErbB2 positive breast cancer (6,7,8,9).

Understanding the post-translational mediated regulation as well as the transcriptional induced in- and outputs of Akt activation will lead to additional valuable therapeutic targets to fight breast cancer.

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